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Age-Dependent Renal Accumulation of 4-Hydroxy-2-Nonenal (HNE)-Modified Proteins Following Parenteral Administration of Ferric Nitrilotriacetate Commensurate with Its Differential Toxicity: Implications for the Involvement of HNE–Protein Adducts in Oxidative Stress and Carcinogenesis

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In this study, we show that the toxicity of ferric nitrilotriacetate (Fe-NTA) can be correlated with the tissue accumulation of 4-hydroxy-2-nonenal (HNE)-modified protein adducts. It is observed that the toxic manifestations of Fe-NTA gradually increase with the increasing age of animals. A dose of Fe-NTA which produces almost 100% mortality in aged rats causes 70% mortality in adults, 30% in pups, 20% in litters, and less than 10% in neonates. The age-dependent increase in its toxicity is also evident from the data of renal microsomal lipid peroxidation and hydrogen peroxide generation. No significant difference in the generation of H_2O_2 and induction of renal microsomal lipid peroxidation between saline- and Fe-NTA-treated neonates, litters, and pups could be observed. However, in adult rats, a significant increase in both of the parameters was observed which was even greater in aged rats. On the contrary, renal glutathione levels in these animals show an inverse relationship with the oxidant generation. In neonates, litters, and pups the maximum decrease of glutathione was up to 22%, whereas in adult and aged rats, the depletion was more than 60% of their respective saline-treated controls. Parallel to this data, blood urea nitrogen and creatinine, the indicators of renal damage, show a significant increase in Fe-NTA-treated adult and aged rats only, whereas no significant alterations were observed in other groups. Similarly, the magnitude of ODC induction and [^{3}H]thymidine incorporation was much higher in aged and adult rats in comparison to other groups of animals after Fe-NTA treatment. Additionally, the immunohistochemical localization studies show a significant increase in HNE-modified protein adducts in kidney of adult and aged rats, whereas no significant staining was observed in other groups. A similar increase in the level of protein carbonyls has also been observed with the increasing age of rats. These data suggest that the toxicity of Fe-NTA increases with the increasing age of rats and correlates with the accumulation of HNE-modified protein adducts. It may also be speculated that Fe-NTA-mediated renal toxicity leading to carcinogenesis may be related to the tissue accumulation of HNE-modified protein adducts. However, further studies are needed to establish a definite role of HNE-modified proteins in Fe-NTA-mediated carcinogenesis.

Key Words: **ageing; carcinogenesis; ferric nitrilotriacetate; 4-hydroxy-2-nonenal; oxidative stress; tumor promotion.**

It has been observed that many chemical compounds which induce tissue oxidative stress also contribute to one or many steps of the multistep process of carcinogenesis following their prolonged and sustained exposure (1). A number of such compounds being food contaminants or food additives are consumed by the human population (2). There are numerous examples of

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such agents. A few of them are discussed in the subsequent part of this introduction. Asbestos, which generates free radicals and augments peroxide-mediated DNA damage (3, 4), is known to cause pulmonary and peritoneal carcinogenesis (5). In skin, a large number of studies have shown that agents which generate oxidative stress such as benzoyl peroxide (BPO)[3] and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) are potent tumor promoters (6, 7). We have shown that cutaneous porphyrin photosensitization produces oxidative stress in skin (8) and that prolonged photosensitization leads to hyperplasia and inflammation (9). It also acts as a weak complete tumor promoter (10). Paraquat, a redox cycling herbicide, produces DNA damage by generating reactive oxygen species (ROS) and has been shown to be a pulmonary tumor promoter (11, 12). Iron overload enhances tissue oxidative stress and augments liver (13), intestinal (14), colonic (15), and pulmonary carcinogenesis (16). Recently, we have shown that iron overload augments dimethylbenz-[*a*]anthracene (DMBA)-induced complete carcinogenesis in murine skin (17). It also enhances TPA- and BPO-mediated cutaneous tumor promotion (18, 19). Potassium bromate, an oxidizing agent used for treatment of wheat flour, acts by generating ROS and producing oxidative modifications in renal DNA and leads to the induction of renal tumor (20). Similarly, a variety of nickel compounds having the potential to generate free radicals are known renal carcinogens (21). Another similar compound is ferric nitrilotriacetate (Fe-NTA), which is formed by the interaction of iron and nitrilotriacetic acid, a known substitute for pyrophosphate used in various kinds of detergents (1). It has been shown to be a complete renal carcinogen. Its prolonged exposure leads to renal proximal tubular necrosis, which is subsequently associated with the high incidence of renal adenocarcinoma in male mice and rats (1, 22). It has also been shown that Fe-NTA enhances diethylnitrosamine-induced renal tumorigenesis (22). Recently, we have shown that it is a potent hepatic as well as renal tumor promoter (23–25). However, the detailed mechanism of its toxicity is not known. Fe-NTA has been shown to generate oxidative stress (26, 27), induce lipid peroxidation, and produce oxidative modifications in DNA (25). One of the major lipid peroxidation products is 4-hydroxy-2-nonenal (HNE), which is highly cytotoxic and readily reacts with crucial biomolecules. It has been shown that HNE degenerates a number of crucial enzymes and leads to mitochondrial dysfunction (28). HNE, generated in kidney of Fe-NTA-exposed animals, has also been speculated to play an important role in Fe-NTA-mediated carcinogenesis. In this study, we, for the first time, show the differential toxicity of Fe-NTA in rats of different age groups and also show that it is commensurate with the differential renal accumulation of HNE–protein adducts. Our results show that the exposure of Fe-NTA tends to produce and accumulate higher amounts of HNE–protein adducts in kidney with the increase in age of animals and could be correlated with the toxicity and carcinogenicity of Fe-NTA in animals of different age groups.

[3] Abbreviations used: Fe-NTA, ferric nitrilotriacetate; HNE, 4-hydroxy-2-nonenal; PMSF, phenylmethylsulfonyl fluoride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PCA, perchloric acid; 2,4-DNPH, 2,4-dinitrophenyl hydrazine; TBA, thiobarbituric acid; TCA, trichloroacetic acid; MDA, malonaldehyde; H_2O_2, hydrogen peroxide; GSH, glutathione; ODC, ornithine decarboxylase; BPO, benzoyl peroxide; TPA, tetradecanoylphorbol 13-acetate; DMBA, dimethylbenz[*a*]anthracene; ROS, reactive oxygen species; IP, intraperitoneal; PMS, postmitochondrial supernatant; BSA, bovine serum albumin; PLC, phospholipase C.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase, phenol red, Tris–HCl, thiobarbituric acid, 2-mercaptoethanol, dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), pyridoxal 5-phosphate, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenylhydrazine (2,4-DNPH), guanidine–HCl, and NTA were purchased from Sigma Chemical Co. (U.S.A.). Diacetylmonoxime, urea, picric acid, sodium tungestate, sodium hydroxide, trichloroacetic acid, and perchloric acid were purchased from CDH (India). [^{14}C]Ornithine (sp activity, 56 mCi/mmol) and [^{3}H]Thymidine (sp activity, 82 Ci/mmol) were purchased from Amersham (U.K.). All other chemicals and reagents used were of highest purity commercially available.

Polyclonal Anti-HNE Antibody

The polyclonal antibody used in this study was raised against HNE-modified KLH and was found to be highly specific to HNE-derived modifications to proteins. It showed no binding to the Michael adducts, NAC-acrolein, -t2P, and -t2N, or to MDA. Its binding required the presence of the 4-hydroxyl group (29), was sensitive to the chain length of the 4-hydroxy-2-alkenal, and recognized mainly cysteine, histidine, and lysine–HNE adducts (22, 29). The epitope recognized by the antibody appears to the hemiacetal form of the HNE-derived portion of protein–HNE adducts (29 and references therein).

Preparation of Fe-NTA Solution

Fe-NTA solution was prepared by the method of Awai *et al.* as described by Iqbal *et al.* (28, 30). Briefly, for the preparation of Fe-NTA, ferric nitrate solution was mixed with the fourfold molar excess of disodium salt of NTA and the pH was adjusted to 7.4 with sodium bicarbonate. The solution was prepared fresh immediately before its use as described earlier.

Experimental Protocol

Animals and treatments. For various sets of biochemical studies, animals (Wistar male rats, except neonates and litters whose sex could not be determined) of five age groups, namely, neonates (1 day old), litters (7 days old), pups (21 days old), adults (120 days old), and aged (400 days old) (Jamia Hamdard Central Animal House Colony) were used throughout the study. Animals were kept on the standard

laboratory feed (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*.

Animals were injected with saline or Fe-NTA intraperitoneally. For studying the effect of Fe-NTA on the survival of rats of various age groups, animals were divided into five different groups (group I, neonates; group II, litters; group III, pups; group IV, adults; group V, aged). Each group was further divided into two subgroups having 10 animals each. Animals of subgroup I received saline and served as respective controls, whereas animals of subgroup II received an ip injection of Fe-NTA at a dose level of 12 mg Fe/kg body wt. The dose regimen of 12 mg Fe/kg body wt is based on the fact that this dose causes 60–70% mortality in adult rats. These animals were observed up to day 14 for the record of their survival. The mortality was recorded in all the groups two times per day, i.e., morning and evening.

For studying the effect of Fe-NTA-mediated generation of renal oxidative stress and renal damage in rats of various age groups, animals were divided into five different groups exactly as mentioned above. Each group was further divided into two subgroups having six animals each. Animals of subgroup I received saline and served as respective controls, whereas animals of subgroup II received an ip injection of Fe-NTA at a dose level of 9 mg Fe/kg body wt. Just before the killing, blood of these animals was collected in separate test tubes. All these animals were killed 12 h after the treatment of saline or Fe-NTA within a span of 1 h.

For studying the effect of Fe-NTA-mediated renal ODC induction, the animal treatment protocol and dose regimen were the same as those described for studying renal oxidative stress. All these animals were killed by cervical dislocation 12 h after the treatment of saline or Fe-NTA within a span of 1 h. Their kidneys were quickly removed and processed for the assay of ODC activity.

Similarly, for studying the effect of Fe-NTA-mediated [^{3}H]thymidine incorporation in renal DNA, the animal treatment protocol and dose regimen were the same as those described for studying ODC activity measurement. However, all of these animals received an ip injection of [^{3}H]thymidine 18 h after the treatment of saline or Fe-NTA. After 2 h of administration of [^{3}H]thymidine, these animals were killed by cervical dislocation within a span of 1 h. Their kidneys were quickly removed, cleaned free of extraneous materials, and homogenized in distilled water for further processing and separation of DNA.

For studying the effect of Fe-NTA-mediated induction of HNE-modified proteins in kidney of rats of various age groups (using immunohistochemical localization techniques), animals were divided into five different groups as described previously. Each group was further divided into two subgroups having six animals each. Animals of each subgroup I received saline and served as respective controls, whereas animals of subgroup II received an ip injection of Fe-NTA at a dose level of 12 mg Fe/kg body wt. The selection of dose of 12 mg Fe/kg body wt in Fe-NTA for conducting immunohistochemical localization studies is based on the previous studies of Toyokuni *et al.* (31), who showed a substantial accumulation of HNE-modified proteins in adult rats at this dose level 1 h after the parenteral administration of Fe-NTA. Thus, in this study, animals were also killed by cervical dislocation 1 h after the treatment of saline or Fe-NTA for the detection of HNE-modified proteins.

For studying the effect of Fe-NTA-mediated induction of protein carbonyl in kidney of rats of various age groups, animals were divided into five different groups as described previously. Each group was further divided into two subgroups having six animals each. Animals of each subgroup I received saline and served as respective controls, whereas animals of subgroup II received an ip injection of Fe-NTA at a dose level of 12 mg Fe/kg body wt. The selection of a dose of 12 mg Fe/kg body wt in Fe-NTA for the estimation of renal protein carbonyl is based on the previous studies of Toyokuni *et al.* (31). Accordingly, animals were killed by the cervical dislocation 3 h after the treatment of saline or Fe-NTA for the estimation of protein carbonyl.

Biochemical Assays

Postmitochondrial supernatant, cytosol, and microsomes preparation. Animals were killed after saline or Fe-NTA treatment. Their kidneys were quickly removed, perfused immediately with ice-cold saline (0.85% sodium chloride), and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%) using a Potter Elvehjem homogenizer. The homogenate was filtered through a muslin cloth and was centrifuged at 800*g* for 5 min at 4°C in an Eltek Refrigerated Centrifuge (Model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 10,500*g* for 20 min at 4°C to get postmitochondrial supernatant (PMS), which was used as a source of enzymes. A portion of the PMS was centrifuged in an Ultracentrifuge (Beckman, L7-55) at 10,5000*g* for 60 min at 4°C to obtain cytosol and microsomes. The pellet was washed with phosphate buffer (0.1 M, pH 7.4) containing potassium chloride (1.17%). This pellet was considered to be the microsomal fraction and was suspended in phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%).

Estimation of reduced glutathione. Reduced glutathione in kidney was assayed by the method of Jollow *et al.* (32). PMS (1.0 ml, 10%) was precipitated with sulfosalicylic acid (1.0 ml, 4%). The samples were kept at 4°C for at least 1 h and then subjected to centrifugation at 1200*g* for 15 min at 4°C. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4), and 0.2 ml DTNB (40 mg/10 ml of phosphate buffer, 0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model-21D).

Lipid peroxidation. The assay for microsomal lipid peroxidation was done following the method of Wright *et al.* (33). The reaction mixture in a total volume of 1.0 ml contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsome, 0.2 ml ascorbic acid (100 mM), 0.02 ml ferric chloride (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1.0 ml TCA (10%). Following addition of 1.0 ml TBA (0.67%), all the tubes were placed in a boiling water bath for a period of 20 min. In the end, the tubes were shifted to an ice bath and then centrifuged at 2500*g* for 10 min. The amount of malonaldehyde formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm using spectrophotometer (Milton Roy 21 D) against a reagent blank. The results were expressed as the nmol MDA formed/h/g tissue at 37°C using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Hydrogen peroxide assay. Hydrogen peroxide (H_2O_2) was assessed by H_2O_2-mediated horse radish peroxidase-dependent oxidation of phenol red by the method of Pick and Keisari (34), with a slight modification. Microsomes (2.0 ml) were suspended in 1.0 ml of solution containing phenol red (0.28 nM), horse radish peroxidase (8.5 units), dextrose (5.5 nM), and phosphate buffer (0.05 M, pH 7.0) and were incubated at 37°C for 60 min. The reaction was stopped by addition of 0.01 ml of NaOH (10 N) and then centrifuged at 800*g* for 5 min. The absorbance of the supernatant was recorded at 610 nm against a reagent blank. The quantity of H_2O_2 produced was expressed as nmol H_2O_2/gm tissue/h based on the standard curve of H_2O_2-oxidized phenol red.

Ornithine decarboxylase activity. ODC activity was determined utilizing 0.4 ml renal 105,000*g* supernatant fraction per assay tube by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine by the method of O'Brien *et al.* (35), as described by Athar *et al.* (6). The kidneys were homogenized in Tris–HCl buffer (pH 7.5, 50 mM) containing EDTA (0.1 mM), pyridoxal phosphate (0.1 mM), PMSF (1.0 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (0.1 mM), and Tween 80 (0.1%) at 4°C using polytron homogenizer (Kinematica

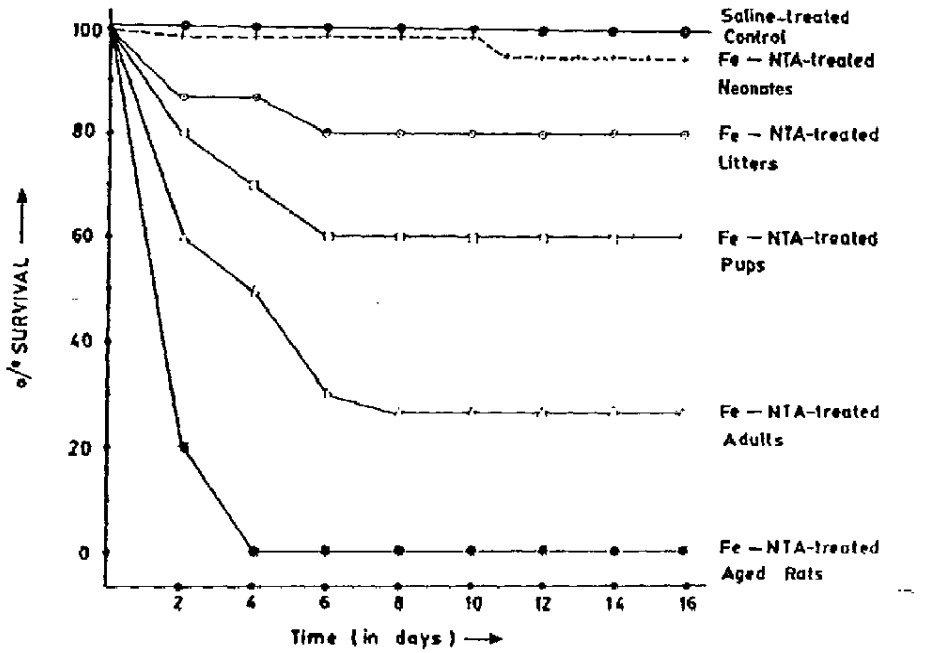


FIG. 1. Effect of Fe-NTA on percentage of survival in rats of various age groups. Dose regimen and treatment protocol are described in the text.

AGPT 3000). In brief, the reaction mixture contained 400 μl enzyme and 0.095 ml cofactor mixture containing pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), brig 35 (0.02%), and [^{14}C]ornithine (0.05 μCi) in a total volume of 0.495 ml. After adding buffer and cofactor mixture to the blank and the other test tube, the tubes, containing 0.2 ml ethanolamine and methoxyethanol mixture in the central well, were covered immediately with a rubber cork and kept in a water bath at 37°C. After 1 h of incubation, the enzyme activity was arrested by injecting 1.0 ml of citric acid solution (2.0 M) along the sides of glass tubes, and the incubation was continued for 1 h to ensure complete absorption of $^{14}CO_2$. Finally, the central well was transferred to a vial containing 2 ml of ethanol and 10 ml toluene-based scintillation fluid was added to it, followed by counting of the radioactivity in a liquid scintillation counter (LKB-Wallace-1410). ODC activity was expressed as pmol $^{14}CO_2$ released/h/mg protein.

Renal DNA synthesis. The isolation of renal DNA and incorporation of [^{3}H]thymidine in DNA was done by the method of Smart *et al.* (36), as described by Iqbal *et al.* (26, 27). Their kidneys were quickly removed and cleaned free of extraneous material, and homogenate (10%, w/v) was prepared in ice-cold water. The precipitate thus obtained was washed with cold TCA (5%) and incubated with cold PCA (10%) at 4°C overnight. After the incubation, it was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in the warm PCA (10%) followed by its incubation in a boiling water bath for 30 min and filtered through Whatman 50. The filtrate was used for ^{3}H counting in a liquid scintillation counter (LKB-Wallace-1410) by adding the scintillation fluid. The amount of [^{3}H]thymidine incorporated was expressed as DPM/μg DNA.

Immunohistochemistry. The immunohistochemical studies were conducted using the avidin–biotin complex method of Hsu *et al.* (37). Frozen sections were postfixed with Bouin's solution for 5 min to suppress endogenous biotin activity. Incubation in 0.09% hydrogen peroxide in 30% (v/v) methanol for 30 min was done for the inhibition of endogenous peroxidase activity. After these procedures, the normal goat serum (Dako, diluted to 1:75) for the inhibition of nonspecific binding of secondary antibody, partially purified rabbit polyclonal antibody against HNE-modified proteins (0.5–2.0 μg/ml), biotin-labeled goat anti-rabbit IgG serum, and avidin–biotin complex were sequentially used. Procedures using normal rabbit serum instead of antibody against HNE-modified proteins showed no or negligible positivity.

Estimation of protein carbonyl. Protein carbonyl was estimated by the method of Levine *et al.* (38). A 0.5-ml aliquot (10%, w/v) of renal 105,000*g* cytosolic fractions was treated with an equal volume of 2,4-DNPH (0.1%) in 2 N HCl and incubated for 1 h at room temperature. This mixture was treated with 0.5 ml trichloroacetic acid (10%, w/v), and after centrifugation the precipitate was extracted three times with ethanol/ethyl acetate (1/1, v/v). The protein sample was then dissolved in 2 ml solution containing guanidine hydrochloride (8 M)/EDTA (13 mM)/Tris (133 mM) (pH 7.2) and UV absorbance was measured at 365 nm. The results were expressed as nmol 2,4-DNPH incorporated/mg protein based on the molar extinction coefficient of 21.0 mM^{-1} cm^{-1}.

Estimation of creatinine and blood urea nitrogen. Creatinine was estimated by the alkaline picrate method of Hare (39). Protein-free filtrate was prepared. In 1.0 ml of plasma/serum, 1.0 ml of sodium tungstate (5%), 1.0 ml of sulfuric acid (0.6 N), and 1.0 ml of distilled water were added, mixed thoroughly, and centrifuged at 800*g* for 5 min. The supernatant was added to a mixture containing 1.0 ml of picric acid (1.05%) and 1.0 ml of sodium hydroxide (0.75 N). The absorbance was recorded exactly after 20 min at 520 nm.

Blood urea nitrogen was estimated by the diacetyl monoxime method of Kanter (40). Protein-free filtrate was prepared. To 0.5 ml of protein free filtrate, 3.5 ml of distilled water, 0.8 ml of diacetyl-monoxime (2%), and 3.2 ml of sulfuric acid–phosphoric acid reagent (the reagent was prepared by mixing 150 ml of 85% phosphoric acid with 140 ml of water and 50 ml of conc H_2SO_4) were added. The reaction mixture was placed in a boiling water bath for 30 min and then cooled. The absorbance was recorded at 480 nm.

Protein in all the samples was determined by the method of Lowry *et al.* (41), using BSA as a standard.

Statistical analysis. The levels of significance between different groups are based on Dunnett's *t*-test followed by an analysis of variance test.

RESULTS

Effect of Fe-NTA Administration on the Percentage of Survival of Rats of Different Age Groups

The effect of Fe-NTA administration on the percentage of survival of rats of various age groups is shown in Fig. 1. Fe-NTA administration decreased the percentage of survival with the increasing age of animals. Treatment of Fe-NTA at a dose level of 12 mg Fe/kg body wt in Fe-NTA resulted in 90, 80, 70, and 30% survival among neonates, litters, pups, and adult rats, respectively, whereas the same dose of Fe-NTA treatment in the case of aged rats proved fatal and almost all animals died (survival ~10%). Fe-NTA administration was well tolerated by neonates and litters only. However, in the case of pups, adults, and aged rats mortality significantly increased gradually. No significant changes in the body, liver, and kidney weights were observed in Fe-NTA-treated rats of all age groups compared to their respective saline-treated controls (data not shown).

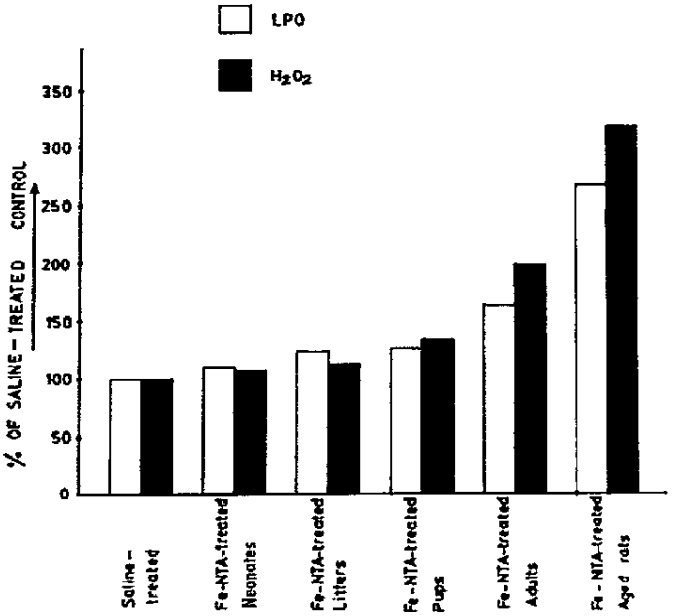


FIG. 3. Effect of Fe-NTA on the enhancement of renal microsomal lipid peroxidation and generation of hydrogen peroxide in rats of various age groups. Value represent means of six animals. The detailed treatment protocol is provided in the text.

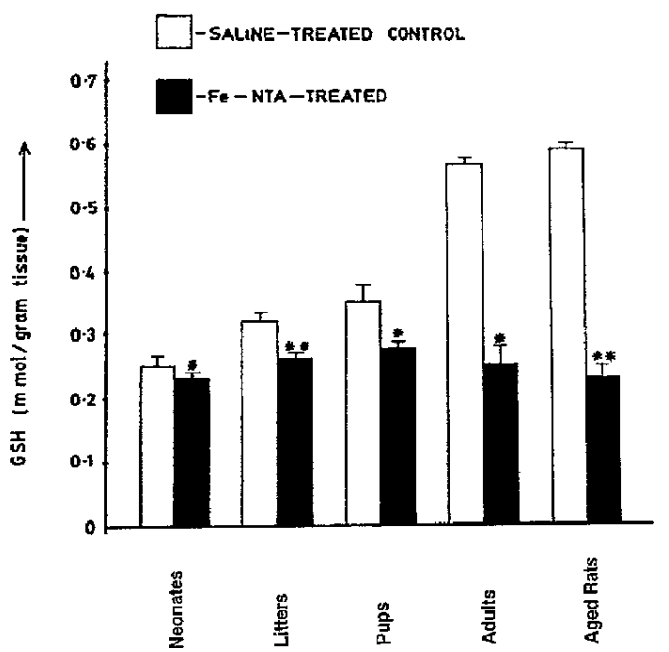


FIG. 2. Effect of Fe-NTA on the depletion of renal glutathione level in rats of various age groups. Values represent means ± SE of six animals. Dose regimen and treatment protocol are described in the text. *Significantly different ($P < 0.05$) when compared with saline-treated control. **Significantly different ($P < 0.001$) when compared with saline-treated control.

Effect of Fe-NTA Administration on the Level of Renal Glutathione in Rats of Different Age Groups

The effect of Fe-NTA administration on the level of renal glutathione in rats of various age groups is shown in Fig. 2. Fe-NTA administration led to a depletion in the level of renal glutathione in rats of various age groups. In neonates, litters, pups, adults, and aged rats, the level of glutathione was 90, 82, 78, 44, and 41%, respectively, of their respective saline-treated controls. Thus, it is clear that this decrease in the level of glutathione was significantly higher in adult and aged rats compared to neonates, litters, and pups. However, in both groups the depletion in glutathione reached a critically low level.

Effect of Fe-NTA Administration on Renal Microsomal Lipid Peroxidation and Generation of Hydrogen Peroxide in Rats of Different Age Groups

The effect of Fe-NTA administration on the enhancement in renal microsomal lipid peroxidation and generation of hydrogen peroxide in animals of various age groups is shown in Fig. 3. Fe-NTA administration led to an enhancement of both renal microsomal lipid peroxidation and generation of hydrogen peroxide in rats of various age groups. The renal microsomal lipid peroxidation in neonates, litters, pups, adults, and aged rats increased respectively to about 110, 120, 124, 163,

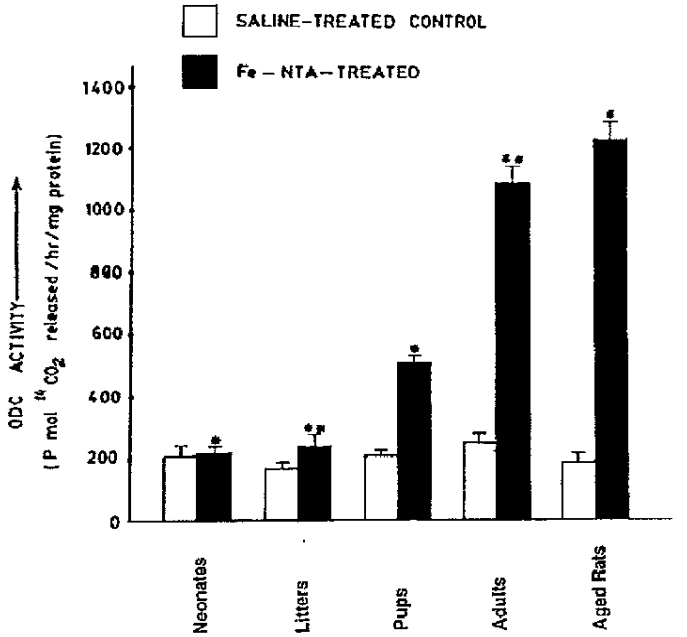


FIG. 4. Effect of Fe-NTA on the induction of renal ornithine decarboxylase activity in rats of various age groups. Value represent means ± SE of six animals. Dose regimen and treatment protocol are described in the text. *Significantly different ($P < 0.05$) when compared with saline-treated control. **Significantly different ($P < 0.001$) when compared with saline-treated control.

and 267%, whereas the enhancement in H_2O_2 generation was about 107, 108, 120, 197, and 317% of their respective saline-treated controls.

Effect of Fe-NTA Administration on Renal ODC Activity in Rats of Different Age Groups

The effect of Fe-NTA administration of on the induction of renal ODC activity in rats of various age groups is shown in Fig. 4. In neonates, litters, and pups, the renal ODC activities increased, respectively, to a value of 103, 136, and 245% of their corresponding saline-treated controls. However, in adults and aged rats, the induction in renal ODC activity was 524 and 619%, respectively, of their corresponding saline-treated controls. Thus, the susceptibility for the induction of renal ODC activity in response to Fe-NTA administration increased with the increasing age of animals and was distinctly pronounced in adult and aged animals.

Effect of Fe-NTA Administration on [^{3}H]Thymidine Incorporation in Renal DNA of Rats of Different Age Groups

The effect of Fe-NTA administration on [^{3}H]thymidine incorporation in renal DNA of rats of various age groups is shown in Fig. 5. Fe-NTA administration resulted in a significant enhancement in [^{3}H]thymidine incorporation in renal DNA of animals of various age groups. The magnitude of enhancement of [^{3}H]thymidine incorporation was much higher in aged rats compared to the animals of other age groups. The enhancement of [^{3}H]thymidine incorporation in DNA was about 1.1-, 1.1-, 1.3-, 4.3-, and 5.8-fold in neonates, litters, pups, adults, and aged rats, respectively, compared to their corresponding saline-treated controls.

Effect of Fe-NTA Administration on the Tissue Content of HNE-Modified Proteins in Rats of Different Age Groups

The effect of Fe-NTA administration on the tissue level of HNE-modified proteins in rats of different age groups as determined by the immunohistochemical localization studies is shown in Figs. 6 and 7. One hour after the administration of Fe-NTA in neonates and litters, the HNE-modified proteins were undetectable in kidney of Fe-NTA-treated animals (Figs. 6a′ and 6b′) and the levels were quite comparable with their respective saline-treated controls (Figs. 6a and 6b) as ascertained by the polyclonal antibodies–tissue inter-

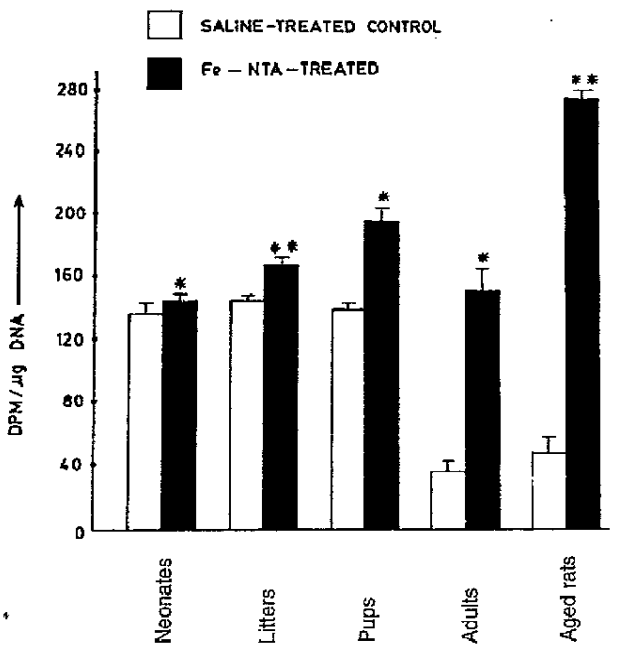


FIG. 5. Effect of Fe-NTA on the enhancement of [^{3}H]thymidine incorporation in renal DNA in rats of various age groups. Values represent means ± SE of six animals. Dose regimen and treatment protocol are described in the text. *Significantly different ($P < 0.05$) when compared with saline-treated control. **Significantly different ($P < 0.001$) when compared with saline-treated control.

action studies. However, treatment of pups with Fe-NTA resulted in the visible appearance of HNE-modified proteins in renal tubular epithelium (Fig. 6c'). In saline-treated control pups, HNE-modified proteins could not be detected under the similar conditions (Fig. 6c). Similarly, an enhanced accumulation of HNE-modified proteins was detected in kidneys of Fe-NTA-treated adult (Figs. 7a and 7b) and aged (Figs. 7d and 7e) rats, which was significantly enhanced with the increasing age of animals. Thus, the expression and accumulation of HNE-modified proteins were found in following order: aged rats >>> adult rats >> pups ~~ litters ~ neonates.

Figure 7 demonstrates the immunohistochemical localization of these modified proteins in the form of yellowish brown reaction product in the cytoplasm of renal epithelium of convoluted tubules in adult (Figs. 7a and 7b) and aged (Figs. 7d and 7e) rats. These protein adducts were also detected in the epithelium undergoing necrotic changes in the form of patchy reaction. The tubular lumen contains a small amount of this protein, suggesting that the HNE-modified proteins are released in the tubular lumen possibly for its elimination in urine. The HNE-modified proteins are primarily detectable in cytoplasm; however, their intranuclear localization could not be ruled out completely. In saline-treated control adult rats (Fig. 7c), these proteins were either absent or detected as mild intracytoplasmic reaction products in some renal tubular epithelium. When the accumulation of HNE-modified proteins in response to Fe-NTA-treatment was compared between adult and aged rats, the modified proteins were found to be accumulated more in the kidney epithelium of Fe-NTA-treated aged rats (Figs. 7d and 7e) compared to adult rats (Figs. 7a and 7b). The saline-treated aged rats also showed intracytoplasmic expression of this protein in the tubular epithelium albeit with low intensity, suggesting that the age-related enhancement of autoperoxidation of renal lipids also leads to the tissue accumulation of HNE-modified protein adducts (Fig. 7f).

Effect of Fe-NTA Administration on Renal Protein Carbonyl in Rats of Different Age Groups

The effect of the administration of Fe-NTA on the induction of renal protein carbonyl in rats of various age groups is shown in Fig. 8. In neonates, litters, and pups, the renal protein carbonyl increased, respectively, to a value of 101, 105, and 119% of their corresponding saline-treated controls. However, in adult and aged rats, the induction was 134 and 170%, respectively, of their corresponding saline-treated controls. Thus, the susceptibility for the induction of renal protein carbonyl in response to Fe-NTA administration increased with the increasing age of animals and was more pronounced in adult and aged animals.

Effect of Fe-NTA Administration on Serum Creatinine and Blood Urea Nitrogen in Rats of Different Age Groups

Fe-NTA administration to rats of various age groups led to a differential enhancement in the value of serum creatinine and blood urea nitrogen as shown in Fig. 9. Blood urea nitrogen and serum creatinine, known markers of renal damage, significantly increased only in adult and aged rats, whereas no significant alterations could be recorded in other age group animals. In adult and aged rats, creatinine increased to 348 and 466%, whereas there was an enhancement in the value of blood urea nitrogen to 393 and 495% of their respective saline-treated controls at a dose level of 9 mg Fe/kg body wt. Thus, the aged rats were more susceptible to kidney damage compared to adult rats at the same dose of Fe-NTA and younger animals seem to be resistant to Fe-NTA toxicity.

DISCUSSION

A number of studies have demonstrated a close association between the induction of lipid peroxidation and the incidence of carcinogenesis in Fe-NTA-exposed animals. These studies also show that membrane lipid peroxidation is one of the basic mechanisms of Fe-NTA-induced renal injury (42, 43). Lipid peroxidation leads to the production of various cytotoxic aldehydes such as MDA and HNE. However, HNE is the major lipid peroxidation product which is highly reactive and most cytotoxic, and hence was selected in this study to assess its role in the age-dependent toxicity of Fe-NTA.

It has been shown that the HNE-modified proteins are accumulated in renal proximal tubules of Fe-NTA-treated rat (31). Using the same polyclonal antibody directed against the HNE-modified proteins, we, in this study, also observed that in Fe-NTA-exposed adult rats there is a substantial accumulation of HNE-modified proteins in the renal proximal tubules along with an increased protein carbonyl titer. However, in neonates and litters, there was no accumulation of HNE-modified proteins. Its distinct accumulation was recorded in pups which gradually increased in adults, reaching a maximal level in aged rats. The observation that the accumulation of HNE-modified proteins following exposure to Fe-NTA critically depends on the age of animals in the present study suggests a close association between the accumulation of these adducts and differential manifestation of Fe-NTA toxicity in animals of different age groups. Additionally, in saline-treated aged animals also, the staining for HNE-modified proteins was observed, suggesting that the aged animals

are more susceptible for the accumulation of HNE-modified proteins. The detailed reasons for the accumulation of HNE-modified proteins in aged rats are not clearly understood. However, it has been shown that the metabolic degradation of exogenously added HNE is reduced with the increasing age of animals (47).

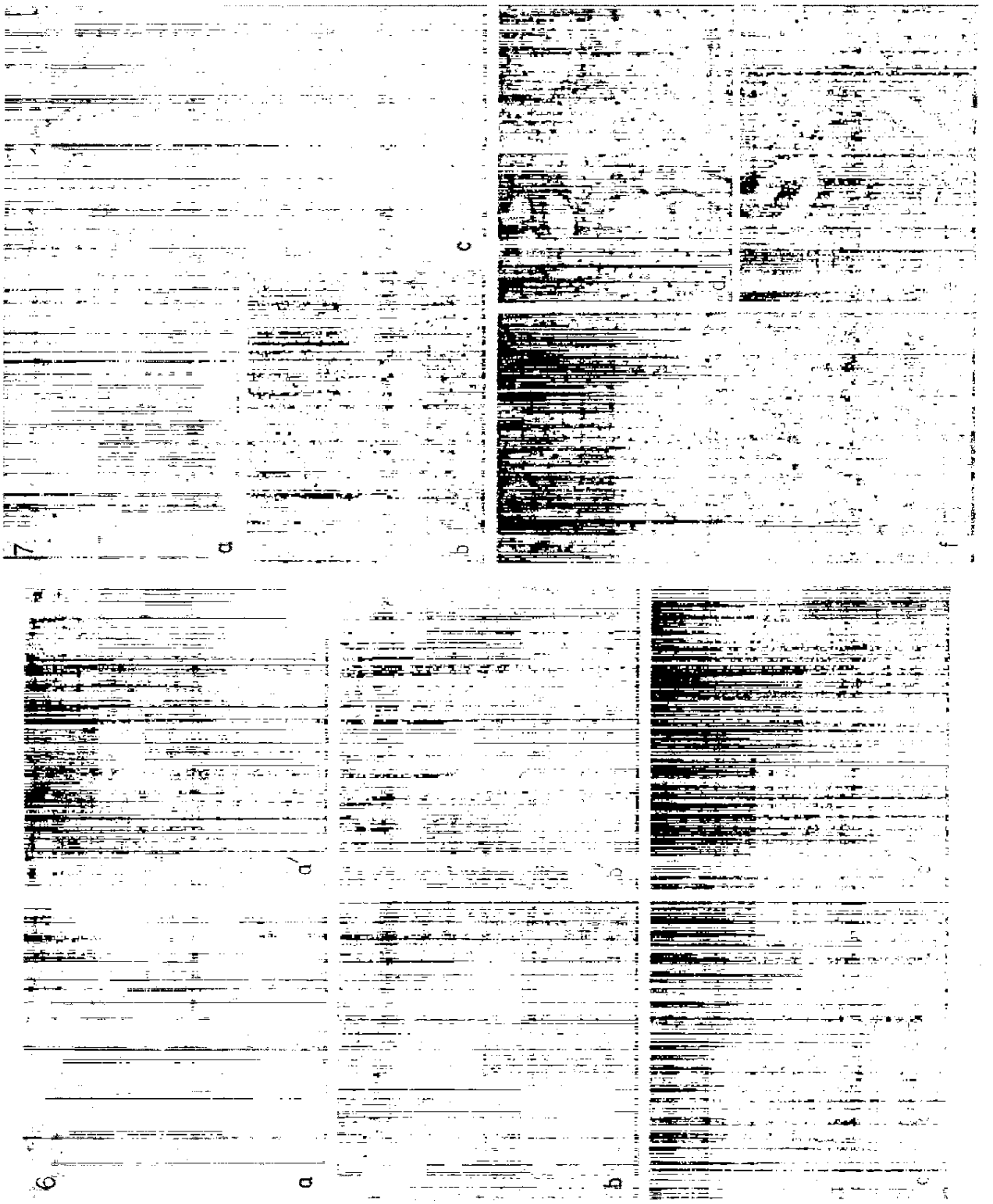
The exact nature of HNE-modified proteins that are accumulated in kidney is also not known. However, HNE has been shown to react with a variety of amino acid residues such as histidine (45), tyrosine (46), lysine (44, 46), cysteine (44, 46), and serine (46) of proteins exhibiting a variety of cytopathological and genotoxic effects (44). The sulfhydryl groups of cysteine residue undergo a Michael addition-type reaction to the L,β-unsaturated bond of HNE to yield thioether derivatives having higher biological half-lives than HNE itself. Similarly, lysine and histidine residues of the protein also undergo Michael-type reactions, leading to HNE–protein adducts such that the aldehyde functional group is preserved (45).

We also conducted further studies to test the hypothesis that the toxicity of Fe-NTA is because of the generation and tissue accumulation of HNE–protein adducts. Surprisingly, in our mortality experiments the dose of Fe-NTA that causes 60–70% mortality in adult rats caused less than 10% mortality in neonates, about 20% mortality in litters, and 30% mortality in pups. However, it produced 100% mortality in aged rats, suggesting that HNE-modified proteins might be involved in the manifestation of Fe-NTA-mediated toxicity. Similarly, in order to test whether a correlation exists between the accumulation of HNE–protein adducts and kidney function in these animals, we have estimated serum creatinine and blood urea nitrogen in rats of all age groups and observed that the value of these two parameters was not significantly different in neonates, litters, and pups compared to their respective saline-treated controls, whereas a multifold enhancement in the values of serum creatinine and blood urea nitrogen was observed in adult and aged rats. These results suggest that the presence of HNE-modified proteins in kidney of Fe-NTA-treated rats may be responsible for hampering the normal kidney functions. However, since the amount of Fe-NTA administered to animals was proportional to their body weights, the older animals received a greater amount of Fe-NTA compared to the animals of other age groups. It is, therefore, possible that the higher concentrations of Fe-NTA may be accumulated in kidney of aged animals compared to younger ones, which may be the cause of greater renal damage in older animals as observed in the present investigation. The involvement of oxidative stress and lipid peroxidation in kidney damage is also evident from our previous studies where we haveshown that vitamin E, a lipophilic membrane-bound antioxidant, and garlic oil and nordihydroguaiaretic acid, the herbal antioxidants, attenuate Fe-NTA-mediated kidney damage and toxicity (23, 30).

Glutathione has also been shown to undergo a rapid Michael-type addition to HNE (44). Cadenos *et al.* (48) demonstrated that cellular exposure of HNE leads to a rapid loss of endogenous glutathione. Our observations on renal glutathione in the present study show that in neonates, litters, and pups there was no significant change in the level of glutathione compared to adult and aged rats where the glutathione depleted to about 60% of their respective saline-treated controls. These data authenticate the reported *in vitro* effect of HNE on glutathione depletion (48). The depletion in glutathione may render the tissue susceptible for the oxidative attack, leading to tissue damage with a severe loss of normal kidney function as is observed in the present study (48). The increase in protein carbonyl level parallel to the depletion in renal glutathione provides evidence for the enhanced oxidative attack in glutathione-depleted kidney. Since the maximum accumulation of HNE-modified proteins has been observed to occur in the renal proximal tubules in the present study, it clearly explains why the renal proximal tubules are the major target of Fe-NTA toxicity *in vivo*. It is believed that iron of the Fe-NTA complex is reduced in renal proximal tubules by cysteine generated by the *in situ* degradation of glutathione. As a result, the ferrous complex becomes weakly bound, releasing redox active iron (49) that can catalyze free radical reactions. The presence of redox active iron has also been demonstrated in serum of Fe-NTA-treated rats (50). Thus, renal proximal tubules become a major site of Fe-NTA-mediated damage. In our previous studies, we have shown that the activities of antioxidant enzymes are reduced in Fe-NTA-treated adult rats which may

FIG. 6. Sections of kidneys from neonates, litters, and pups studied for the localization of HNE-modified proteins. The proteins were undetectable in Fe-NTA-treated neonates (a′) and litters (b′). Saline-treated control, neonates (a), and litters (b) also had no such proteins detectable in kidney. Minimal immunoreactive HNE-modified proteins could be seen in the cytoplasm of renal tubular cells of pups treated with Fe-NTA (c′). The proteins could not be detected in saline-treated control pups (c). a–c and a′–c′: ×400. a–c and a′–c′: Avidin–biotin complex peroxidase method.

FIG. 7. Sections of kidneys from Fe-NTA-treated adult and aged rats stained for localization of HNE-modified proteins. Moderate intracytoplasmic yellowish–brown reaction products were detected in the cytoplasm of renal epithelium including the cells necrosed in both adult (a, b) and aged (d, e) rats as localized by the rabbit polyclonal antiserum. Saline-treated control adult (c) and aged (f) rats had minimal or an absence of these proteins in renal epithelium. a–f: ×200. a–f: Avidin–biotin complex peroxidase method.



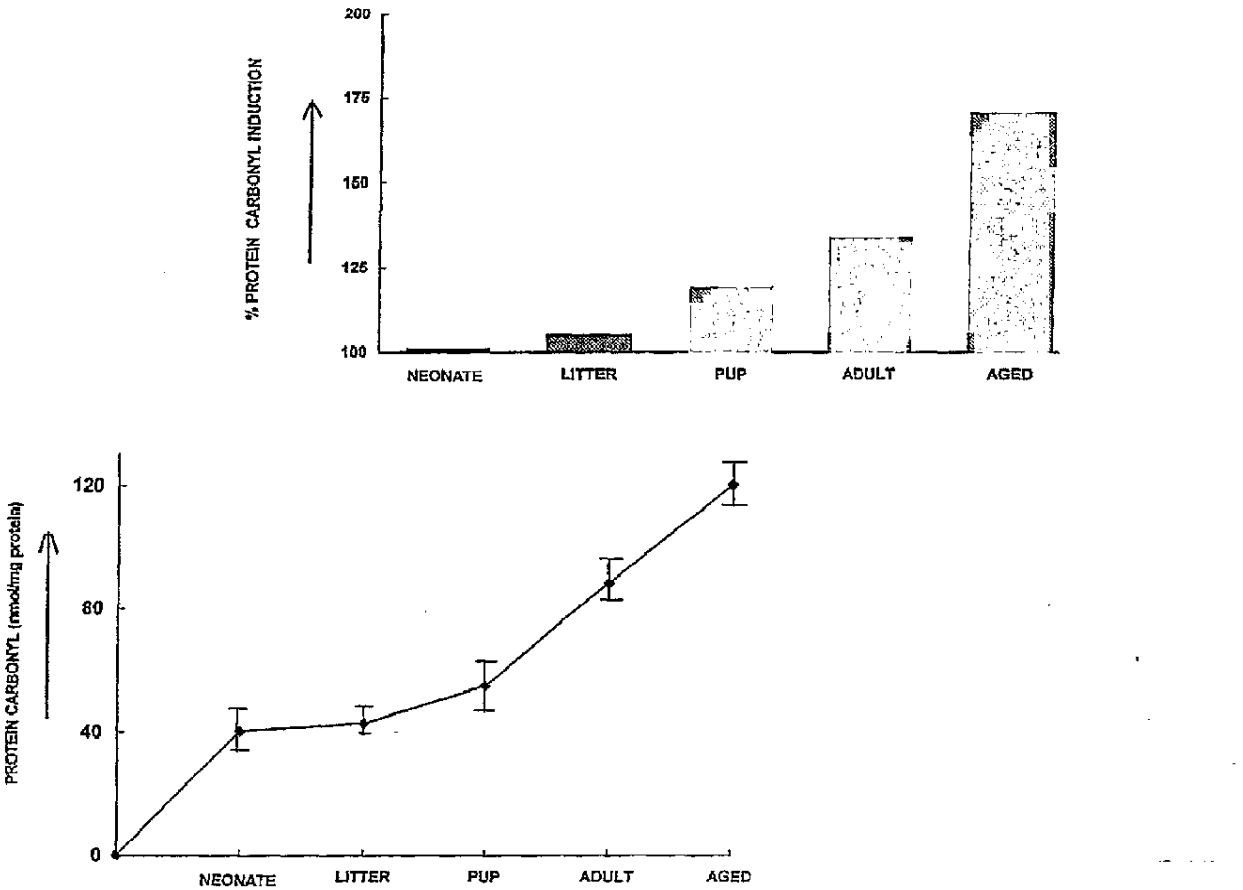


FIG. 8. Effect of Fe-NTA on the induction of renal protein carbonyl in rats of various age groups. (Inset) Percentage of induction of renal protein carbonyl in the rats of various age groups compared to their corresponding saline-treated controls. Value represent means ± SE of six animals. Dose regimen and treatment protocol are described in the text.

be responsible for further augmenting the oxidant-mediated injury in Fe-NTA exposed rats (26–28).

Similar to the observed effect of Fe-NTA on mortality and kidney function, Fe-NTA-mediated inducibility of ODC activity and [^{3}H]thymidine incorporation in renal DNA were also dependent on the age of animals. No significant differences in the ODC inducibility and [^{3}H]thymidine incorporation in renal DNA of Fe-NTA- and saline-treated neonates were observed, but a gradual increase with the age of animals in both of these parameters was recorded. Maximal effects were observed in aged rats. ODC, a rate-limiting enzyme of polyamine biosynthesis, yields polyamines that stimulate the synthesis of DNA. In several experimental models, ODC has been shown as a marker of cell proliferation and tumor promotion (51). Recently, it has been reported that overexpression of ODC plays an important role in carcinogenesis (52). We have also observed an increase in hepatic and renal ODC activity and [^{3}H]thymidine incorporation in DNA, as well as in renal tumor promotion following the parenteral administration of Fe-NTA in adult animals (26, 27). Furthermore, in the present study, we provide a direct correlation between the HNE-modified protein accumulation and cellular proliferation responses in kidney of animals of different age groups. Based on these results, it may be suggested that the susceptibility for Fe-NTA-mediated tumor promotion/tissue proliferation may be increased with the increasing age of animals. Similar to various known tumor promoters, HNE (in the submicromolar concentration range) was shown to be chemotactic and stimulated phospholipase C (PLC) activity (53). Parallel to its effect on chemotaxis and PLC activation, the observed high inducibility of ODC activity and enhanced [^{3}H]thymidine incorporation in aged rats may be due to the enhanced generation of HNE (31).

In addition, the role of oxidative DNA damage which is apparent from the high level of 8-OH-dG in Fe-NTA-treated tumor-bearing animals cannot be ruled out (31,

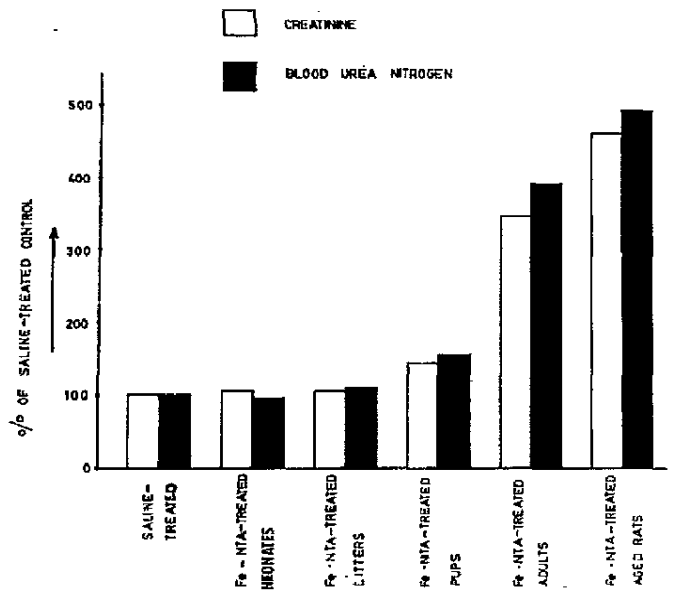


FIG. 9. Effect of Fe-NTA on the enhancement of serum creatinine and blood urea nitrogen in rats of various age groups. Values represent means of six animals. Dose regimen and treatment protocol are described in the text.

54). The role of HNE-modified proteins in the 8-OH-dG formation has not so far been demonstrated. However, it has been shown that lipid peroxidation provokes 8-OH-dG formation (55) and that Fe-NTA enhances 8-OH-dG formation only in kidney, leaving an open possibility that HNE formation may be a link between the lipid peroxidation and the oxidative DNA damage (31). The observations in the present study indicate an increased production of H_2O_2 parallel to the enhancement in lipid peroxidation as a function of the age of animals. It is known that Fe-NTA in presence of H_2O_2 stimulates the production of · OH through the Fenton reaction which has been shown to be responsible for the production of 8-OH-dG (56). The · OH production may become more damaging in an oxidizing tissue environment having a low antioxidant status as has been observed in kidney of Fe-NTA-treated animals (23, 24, 26–28). However, further studies are required to understand the exact mechanism of action of Fe-NTA in animals of different age groups. It may be suggested that experiments employing neonates continuously exposed to Fe-NTA until the age of 2 months and studying their susceptibility to kidney damage and renal tumor induction vis-a-vis the study of kinetics of renal accumulation of HNE-modified proteins over a period of 1 year may be helpful in understanding role of HNE-modified proteins in renal damage and renal carcinogenesis. It will be further helpful to design the simulating experiments using HNE and other oxidants which may induce the renal accumulation of HNE-modified proteins and to study the renal damage and carcinogenesis.

In summary, our data indicate that the renal toxicity and carcinogenicity of Fe-NTA can be correlated with the formation and accumulation of HNE–protein adducts in kidney. Additionally, there also seems to be an age-dependent enhancement in the susceptibility of animals to oxidant-induced renal injury.

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REFERENCES

1. Toyokuni, S. (1996) *Free Radical Biol. Med.* **20,** 553–566.
2. Ito, N., Fukushima, S., Hagiwara, A., Shibata, M., and Ogiso, T. (1983) *J. Natl. Cancer. Inst.* **70,** 343.
3. Mahmood, N., Khan, S. G., Athar, M., and Rahman, Q. (1994) *Biochem. Biophys. Res. Commun.* **200,** 687–694.
4. Rahman, Q., Mahmood, N., Khan, S. G., Arif, J. M., and Athar, M. (1997) *Environ. Health Perspect.* **105,** 1109–1112.
5. Mossman, B. T., Bignon, J., Corn, M., Seaton, A., and Gee, J. B. L. (1990) *Science* **247,** 294–301.
6. Athar, M., Raza, H., Bickers, D. R., and Mukhtar, H. (1990) *J. Invest. Dermatol.* **94,** 162–165.
7. Cerutti, P. A. (1985) *Science* **227,** 375–381.
8. Athar, M., Elmets, C. A., Bickers, D. R., and Mukhtar, H. (1989) *J. Clin. Invest.* **83,** 1137–1143.
9. Giri, U., Sharma, S. D., Abdulla, M., and Athar, M. (1995) *Biochem. Biophys. Res. Commun.* **209,** 698–705.
10. Giri, U., Iqbal, M., and Athar, M. (1996) *Carcinogenesis* **17,** 2023–2028.
11. Bojan, F., Nagy, A., and Herman, K. (1978) *Bull. Environ. Contam. Toxicol.* **20,** 573–576.
12. Ali, S., Jain, S. K., Abdulla, M., and Athar, M. (1996) *Biochem. Mol. Biol. Intl.* **39,** 63–67.
13. Smith, A. G., Corthew, P., Francis, J. E., Cabral, J. R., and Menson, M. (1993) *Carcinogenesis* **14,** 1381–1387.
14. Siegers, C. P., Bumann, D., Trepkau, H. D., Schadwink, L. B., and Baretton, G. (1992) *Cancer Lett.* **65,** 245–249.
15. Nelson, R. L. (1992) *Free Radical Biol. Med.* **12,** 161–168.
16. Qian, M., and Eaton, J. W. (1991) *Am. J. Pathol.* **139,** 1425–1434.
17. Rezazdeh, H., and Athar, M. (1997) *Redox Rep.* **3,** 303–309.
18. Rezazdeh, H., Julka, P. K., and Athar, M. (1998) *Skin Pharmacol.* **11,** 98–103.
19. Rezazdeh, H., and Athar, M. (1998) *Cancer Lett.* **126,** 135–142.
20. Kurokawa, Y., Hayashi, Y., Maekawa, A., Takahashi, M., and Kokuba, T. (1982) *Jpn. J. Cancer. Res.* **73,** 335–338.
21. Athar, M., Hasan, S. K., and Srivastava, R. C. (1987) *Biochem. Biophys. Res. Commun.* **142,** 1276–1281.
22. Athar, M., and Iqbal, M. (1998) *Carcinogenesis* **19,** 1133–1139.
23. Iqbal, M., Giri, U., and Athar, M. (1995) *Biochem. Biophys. Res. Commun.* **212,** 557–563.

24. Toyokuni, S., and Sagripanti, J.-L. (1993) *Carcinogenesis* **14,** 228–227.
25. Iqbal, M., Sharma, S. D., Zadeh, H. R., Hasan, N., Abdullah, M., and Athar, M. (1996) *Redox Rep.* **2,** 385–391.
26. Iqbal, M., Rezazadeh, H., Ansar, S., and Athar, M. (1988) *Hum. Exp. Toxicol.* **17,** 163–171.
27. Iqbal, M., and Athar, M. (1998) *Food Chem. Toxicol.* **36,** 485–495.
28. Humphries, K. M., Yoo, Y., and Szweda, L. E. (1988) *Bioxchemistry* **37,** 552–557.
29. Uchida, K., Itakura, K., Kawakishi, S., Hiai, H., Tokokuni, S., and Stadtman, E. R. (1995) *Arch. Biochem. Biophys.* **76,** 107–113.
30. Ansar, S., Iqbal, M., and Athar, M. (1999) *Carcinogenesis* 599–606.
31. Toyokuni, S., Uchida, K., Okamato, K., Nakakuki, Y. H., Hiai, H., and Stadtman, E. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2616–2620.
32. Jollow, D. J., Mitchell, J. R., Zampaglione, N., and Gillete, J. R. (1974) *Pharmacology* **11,** 151–169.
33. Wright, J. R., Colby, H. D., and Miles, P. R. (1981) *Arch. Biochem. Biophys.* **206,** 296–300.
34. Pick, A., and Keisari, Y. (1981) *Cell. Immunol.* **59,** 301–308.
35. O'Brien, T. G., Simsiman, R. C., and Boutwell, R. K. (1975) *Cancer Res.* **35,** 1662–1670.
36. Smart, R. C., Huang, M. T., and Conney, A. H. (1986) *Carcinogenesis* **7,** 1865–1870.
37. Hsu, S., Raine, L., and Fanger, H. (1980) *Am. J. Clin. Pathol.* **75,** 734–738.
38. Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A., Ahn, B., Shaltiel, S., and Stadtman, E. R. (1990) *Methods Enzymol.* **186,** 464–478.
39. Hare, R. S. (1950) *Proc. Soc. Exp. Biol. Med.* **74,** 148.
40. Kanter, M. W. (1975) Clinical Chemistry, p. 80, The Bobbr Merill Co., Inc., USA.
41. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
42. Li, J.-L., Okada, S., Hamazaki, S., Ebina, Y., and Midorikawa, O. (1987) *Cancer Res.* **47,** 1867–1869.
43. Toyokuni, S., Okada, S., Hamazaki, S., Minamiyama, Y., Yamada, Y., Liang, P., Fukunaga, Y., and Midorikawa, O. (1990) *Cancer Res.* **50,** 5574–5580.
44. Esterbauer, H., Schaur, J. S., and Zollner, H. (1991) *Free Radical Biol. Med.* **11,** 81–128.
45. Uchida, K., and Stadtman, E. R. (1993) *J. Biol. Chem.* **268,** 6388–6393.
46. Jurgen, G., Lang, J., and Esterbauer, H. (1986) *Biochem. Biophys. Acta* **875,** 103–114.
47. Chiarpotto, E., Biasi, F., Scavazza, A., Camandola, S., Dianzani, M. U., and Poli, G. (1995) *Biochem. Biophys. Res. Commun.* **207,** 477–484.
48. Cadenas, E., Muller, A., Brigelius, R., Esterbauer, H., and Sies, H. (1983) *Biochem. J.* **214,** 479–487.
49. Okada, S., Minamiyama, Y., Hamazaki, S., Toyokuni, S., and Sotomatsu, A. (1993) *Arch. Biochem. Biophys.* **301,** 138–142.
50. Liu, M., Okada, S., and Kawabata, T. (1991) *Acta Med. Okayama* **45,** 401–408.
51. Boutwell, R. K. (1983) *In* Advances in Polyamine Research (Bachrach, U., Kaye, A., and Chayen, R., Eds.), Vol. 4, pp. 127–134, Raven Press, New York.
52. Shantz, L. M., and Pegg, A. E. (1994) *Cancer Res.* **54,** 2313–2316.
53. Grune, T., Siems, W. G., Zollner, H., and Esterbauer, H. (1994) *Cancer Res.* **54,** 5231–5235.
54. Umemura, T., Sai, K., Takagi, A., Hasegawa, R., and Kurokawa, Y. (1990) *Carcinogenesis* **11,** 345–347.
55. Park, J. W., and Floyd, R. A. (1992) *Free Radical Biol. Med.* **12,** 245–250.
56. Hamazaki, S., Okada, S., Li, J-L., Toyokuni, S., and Midorikawa, O. (1989) *Arch. Biochem. Biophys.* **272,** 10–17.